



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

Combining microscopy and biochemistry to study meiotic spindle assembly in *Drosophila* oocytes

Citation for published version:

Rome, P & Ohkura, H 2018, Combining microscopy and biochemistry to study meiotic spindle assembly in *Drosophila* oocytes. in H Maiato & M Schuh (eds), *Mitosis and Meiosis Part B*. 238454 edn, vol. 145, Methods in Cell Biology, vol. 145, HUMANA PRESS INC, Cambridge, pp. 237-248.
<https://doi.org/10.1016/bs.mcb.2018.03.026>

Digital Object Identifier (DOI):

[10.1016/bs.mcb.2018.03.026](https://doi.org/10.1016/bs.mcb.2018.03.026)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Mitosis and Meiosis Part B

Publisher Rights Statement:

<https://creativecommons.org/licenses/by-nc-nd/4.0/legalcode>

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.



Combining microscopy and biochemistry to study meiotic spindle assembly in *Drosophila* oocytes.

Pierre Romé and Hiroyuki Ohkura

Wellcome Centre for Cell Biology, School of Biological Sciences, University of Edinburgh,
Edinburgh EH9 3BF, UK

Abstract

Studies using *Drosophila* have played pivotal roles in advancing our understanding of molecular mechanisms of mitosis throughout the past decades, due to the short generation time and advanced genetic research of this organism. *Drosophila* is also an excellent model to study female meiosis in oocytes. Pathways such as the acentrosomal assembly of the meiotic spindle in oocytes are conserved from fly to humans. Collecting and manipulating large *Drosophila* oocytes for microscopy and biochemistry is both time- and cost-efficient, offering advantages over mouse or human oocytes. Therefore, *Drosophila* oocytes serve as an excellent platform for molecular studies of female meiosis using a combination of genetics, microscopy and biochemistry. Here we describe key methods to observe the formation of the meiotic spindle either in fixed or in live oocytes. Moreover, biochemical methods are described to identify protein-protein interactions *in vivo*.

Keywords: *Drosophila*, oocyte, spindle, microtubule, immunostaining, RNAi, fluorescent *in situ* hybridization, transgenesis, live-imaging, protein-protein interaction, biochemistry, immunoprecipitation

I. Introduction

During cell division, a spindle made of microtubules is assembled to segregate chromosomes into two equal complements. In mitosis, spindle microtubules emanate mainly from microtubule organising centres, called centrosomes (Kirschner and Mitchison, 1986). Although the basic cell division machinery is conserved between mitosis and meiosis, the latter often involves modifications to molecular pathways (Ohkura et al., 2015). Firstly, in the first meiotic division (meiosis I) homologous chromosomes segregate, which is followed by the segregation of sister chromatids in second meiotic division (meiosis II). Secondly, in most animals, oocytes assemble the meiotic spindle in the absence of centrosomes (Karsenti and Vernos, 2001; McKim and Hawley, 1995), showing the existence of alternative, centrosome-independent pathways for meiotic spindle assembly. Despite being a fundamental biological question with potential medical implication, the assembly of the meiotic spindle remains to this day poorly understood at the molecular level. The vast availability of genetics tools combined with the short generation time make the fruit fly, *Drosophila melanogaster*, an excellent model to study the acentrosomal spindle assembly. Moreover, *Drosophila* oogenesis naturally arrests prior to fertilization in metaphase I (King, 1970; Theurkauf and Hawley, 1992; Page and Orr-Weaver, 1997), allowing the collection of substantial numbers of synchronised oocytes for biochemical and cytological studies.

Transgenesis using transposon-mediated integration, homologous recombination and site-directed integration provides a robust tool to introduce genetic modifications into the *Drosophila* genome (Bateman et al., 2006; Bischof et al., 2007; Fish et al., 2007; Groth et al., 2004; Groth and Calos, 2004). Using transgenesis, the expression of genes coding for tagged proteins enables the visualisation of proteins of interest in live oocytes. Moreover, the transgenes can be placed under the control of a ubiquitous promoter allowing expression in the whole fly, or can be expressed in a tissue-specific manner, using the Gal4 inducible promoter system, restricting their expression to the female germline (Fischer et al., 1988; Rørth, 1998).

A powerful way to understand the role of a gene is to inactivate it and study the induced phenotype. Mutants of many genes are already available. Furthermore, RNA interference (RNAi) *in vivo* enables gene knock-down in specific tissues. Unfortunately, the long hairpin RNAs commonly used to inactivate genes in *Drosophila* are ineffective in female germlines. Instead, a new strategy has been developed based on short hairpin RNAs (shRNA) that proved to efficiently inactivate genes during oogenesis (Ni et al., 2011). RNAi lines effective in germline cells now cover about 59% of the *Drosophila* genome (8240 genes out of ~14000 total coding genes, in October 2017 according to the Harvard TRiP Project). This number keeps growing and custom-made lines can be generated with relative ease. Efficiency of RNAi can be tested by western Blot (see C.i) or by RT-qPCR after ovaries are dissected. The possibility of off-target effects can be excluded by testing phenotypes of multiple non-overlapping shRNAs, or rescuing the phenotype by expressing an RNAi resistant variant which codes for the targeted protein.

The imaging of a meiotic spindle, both in fixed and live oocytes, has always been a challenging task due to the thickness of mature oocytes. Although the development of confocal microscopy overcame the problem to some degree, effective methods for oocyte fixation and permeabilization are nevertheless required to obtain satisfactory images. This chapter will first discuss methods to fix the oocytes in metaphase I to study the localisation of proteins, and then methods to image the meiotic apparatus and proteins in live oocytes. Finally, this chapter will also cover methods to immunoprecipitate proteins of interest in order to study their interacting partners by western blotting or mass spectrometry. Taking advantage of the natural cell cycle arrest in metaphase I and the large size of *Drosophila* oocytes, it is possible to collect a substantial amount of material for biochemical assays. Therefore, *Drosophila* provide a unique opportunity to combine genetics, microscopy and biochemistry to study meiotic spindle assembly in oocytes.

II. Methods

A. Staining the meiotic spindle in fixed oocytes

A.i) Immunostaining of fixed oocyte

A classical approach to understand the function of a gene during a biological process is to characterise the effects of its depletion in comparison to a control. Genetic mutations can be used if they are available. In case a mutation causes lethality, generating germline clones using site specific recombination allows production of phenotypically wild-type flies carrying mutant oocytes. Furthermore, transgenic RNAi lines have been generated at Harvard University (<https://fgr.hms.harvard.edu/fly-in-vivo-rnai>) and are available from the Bloomington Drosophila Stock Center (<http://flystocks.bio.indiana.edu/Browse/RNAi/RNAihome.htm>). Flies expressing shRNA (using pValium 20, 21 and 22; <https://fgr.hms.harvard.edu/gal4-uas-germline>), not long hairpin RNA commonly used in somatic cells, are adequate for depletion in oocytes (Ni et al., 2011). Customised RNAi lines can be generated if required (for more detail see at <https://fgr.hms.harvard.edu/cloning-and-sequencing>). In these lines, shRNAs are placed under the control of UASp promoter that can be activated by Gal4 (Rørth, 1998). The UASp promoter is derived from the UAS_t promoter, and allows for efficient expression in the female germline cells, as well as somatic cells. They are crossed with flies expressing Gal4 under the control of a female germline specific promoter: *nanos-GAL4* (Rørth, 1998), *maternal α -tubulin-GAL4* (Staller et al., 2013) or MTD-Gal4 (Maternal Triple Driver; <https://fgr.hms.harvard.edu/gal4-uas-germline>). In female progeny expressing the shRNA only during oogenesis, the gene of interest will be inactivated specifically in the female germline. About 25 of these females, in parallel to wild-type control flies, are then matured at 25°C in the presence of ~13 males in fly food bottles with dried yeast pellets, as mating and food stimulate oogenesis and ovulation (Bloch Qazi et al., 2003). In 3-5 days at 25°C,

the females produce matured stage-14 oocytes naturally arrested in metaphase I (Gilliland et al., 2009; King, 1970).

As microtubules are highly dynamic, it is crucial to fix oocytes quickly before they are depolymerised. One effective method, which we routinely use in our lab, is to directly dissect ovaries from flies in methanol (Cullen and Ohkura, 2001; Tavosanis et al., 1997). About 4-5 mature females are dropped into methanol in a watch glass. Under a dissection microscope, the abdomens of the mature females are quickly torn apart in methanol with a pair of forceps. The methanol immediately penetrates the ovary tissues and fixes the oocytes. The ovaries are transferred using a Pasteur pipette into a 15 ml tube containing 3-5 ml of fresh methanol. At this stage, oocytes can be stored in methanol for months at -20°C. Oocytes are gently sonicated (37%, 1 second pulse, three times; the settings need to be optimised for the sonicator used and firmness/fragility of ovaries) to remove the chorion but preserve the integrity of the oocyte. This sonication step is essential as the chorion would hinder the penetration of antibodies during the subsequent immuno-staining protocol. The fixed de-chorionated oocytes, distinguished by a more matte appearance than the shiny chorionated oocytes, are gently transferred using forceps or a pipette into a microfuge tube containing fresh methanol. Sonication can be repeated until ~100-150 oocytes are collected.

Fixed oocytes are rehydrated through washes of PBS-methanol (50%-50%) and PBS before a 30-minute incubation in a blocking solution of PBS-T (PBS + 0.1% Triton X-100) + 10% foetal calf serum (FCS). Primary antibodies against the protein of interest are diluted as required in PBS-T + 10% FCS and incubated with the oocytes for 2 hours to overnight. Oocytes are washed three times for 10 minutes with PBS-T then incubated for 2 hours with a fluorophore-conjugated secondary antibody diluted in PBS-T + 10% FCS. Oocytes are extensively washed in PBS-T 4 times for a total of 1 hour before being mounted. DAPI (final 0.5 µg/ml) should be added with the secondary antibody or during the last wash to visualise the chromosomes. Oocytes are transferred onto a paint brush and excess liquid drawn away by a tissue applied against the base of the brush. The oocytes are transferred to the tip of a

pair of fine forceps and then into a 20 μ l drop of mounting medium (85% glycerol, 2.5% propyl gallate) on a coverslip (18x18 mm). Oocytes are gently pushed down with forceps onto the surface of the coverslip. Once they are all in contact with the coverslip, a glass slide is gently pressed against the drop of mounting medium to capture the coverslip by capillarity action. Finally, slides are sealed with nail varnish. Slides can be kept for a few weeks if stored at 4°C.

Slides are imaged using a confocal microscope to visualise the spindle in the oocyte. Oocytes are screened by eye to locate the meiotic chromosomes labelled with DAPI. Only about ~20% of the oocytes are appropriately positioned and oriented to allow imaging at high quality. The spindle or proteins of interest are imaged using a 63x (NA1.4; oil) objective with 100 nm pixel size, and ~1 μ m Z-slice thickness and 0.5 μ m Z-step acquisition. With this method, it is possible to visualise the spindle architecture using a commercial anti- α -tubulin antibody (DM1A, #T6199 SIGMA) and/or the localisation of proteins of interest with antibodies against them. The antibodies against Msps (Mini spindles) or TACC (Transforming Acidic Coiled-Coil) proteins highlight the poles of the meiotic spindle in mature oocytes arrested in metaphase I (Cullen and Ohkura, 2001) and the antibody against Subito highlights the equatorial regions of the spindle (Jang et al., 2005) (Figure 1).

Sometimes, antibodies against proteins of interest are either unavailable or incompatible with immunostaining. It is however possible to generate transgenic flies expressing a tagged protein. The tag type remains at the discretion of the user and the promoter can be chosen to be inducible (UASp), ubiquitous or endogenous. Several commercial antibodies directed against a variety of tags (anti-GFP for example #A-11122 ThermoFisher Scientific) work in *Drosophila* oocytes and have been proven to be efficient in studying the localisation of ectopically expressed, tagged proteins (Beaven et al., 2017). Other tags such as 3xFLAG, Myc, HA or poly-Histidine should work but have not been tested in our lab.

A.ii) Microtubule regrowth assay

Visualising the fixed metaphase I spindle is informative, but it is also possible to follow the regrowth of the microtubules of the spindle after depolymerisation. To do so, matured females can be individually incubated in microfuge tubes on ice for three hours to fully depolymerise the microtubules (Radford et al., 2012). Oocytes are then fixed in cold methanol immediately before regrowth (time point 0), or incubated in a 25°C water bath to allow microtubule regrowth for different periods of time before fixation with methanol at room temperature. The rest of the protocol is the same as the immunostaining described above. After 5 minutes, we observed that the spindle is fully regrown and that bipolarity is roughly re-established (our unpublished data). This method can be used to determine the role of a gene in microtubule regrowth (by using RNAi and mutants, see above).

A.iii) *In situ* hybridization

In meiosis I, kinetochores of sister chromatids are tightly associated together, and kinetochores of homologous chromosomes must be bi-oriented and attached by microtubules emanating from opposite poles. Failure in this process can result in chromosome mis-segregation (McKim and Hawley, 1995; Radford et al., 2017). Kinetochores assemble at a particular region of the chromosome called centromere. Centromeres of each homologous chromosome are pulled towards opposite poles (Dernburg et al., 1996). Pericentromeric heterochromatin contains tandemly repeated sequences, with at least one unique repetitive sequence per chromosome (Abad et al., 1992; Carmena et al., 1993; Dernburg, 2000; Dernburg et al., 1996; Loh et al., 2012; Meireles et al., 2009; Radford et al., 2012; Zhaunova et al., 2016). Therefore, a specific pair of homologous centromeres can be visualised under a fluorescent microscope by fluorescent *in situ* hybridization (FISH) using a fluorescently labelled oligonucleotide that can hybridise to these chromosome-specific sequences (Dernburg, 2000; Dernburg et al., 1996).

To generate a probe, terminal deoxynucleotidyl transferase (TdT) is used to add fluorescently labelled nucleotides to the 3' terminus of an oligonucleotide. 5 pM of an

oligonucleotide is mixed with 0.8 mM of dTTP, 0.1 mM of dUTP conjugated with fluorescent molecules (such as Alexa 546-dUTP) and 1.5 unit/ μ l of terminal deoxynucleotidyl transferase (TdT; #M1871 Promega) in transferase buffer. The solution is incubated at 37°C for one hour before inactivating TdT at 70°C for 10 minutes. Labelled oligonucleotides are separated from unincorporated nucleotides using a G25 gel filtration column (Miniquick column #11814397001 Sigma) and stored at -20°C.

Matured flies are dissected in methanol, ovaries dechorionated by sonication and oocytes rehydrated and collected, in the same way as for immunostaining, described in Section Ai. 20X SSC buffer (3M NaCl, 0.3M tri-sodium citrate, pH=7) is prepared. Oocytes are post-fixed in 200 μ l of PBS + 8% formaldehyde (Paraformaldehyde 32% Solution EM grade #15714 Electron Microscopy Sciences) for 2 minutes at 37°C and rinsed twice in 300-600 μ l of 2X SSCT buffer (10% 20X SSC, 0.1% Tween-20). Oocytes are washed at least 10 minutes in 2X SSCT. They are then washed for 10 minutes in 400 μ l of freshly made 50% formamide buffer (10% 20XSSC, 50% formamide, 0.1% Tween-20) and incubated in 400 μ l of 50% formamide buffer for 1 hour at 37°C. 50% formamide buffer is entirely removed and replaced by 40 μ l of the probe solution made by mixing 4 μ l of the probes with 36 μ l of 1.1X hybridization buffer (16.67% 20X SSC, 55.56% formamide, 0.111 g/ml dextran sulphate sodium salt, pH=7). Oligonucleotides are denatured for 2 minutes at 91°C and hybridized from 1 hour to overnight at 30°C. Oocytes are washed twice with 500 μ l of 30°C pre-heated 50% formamide buffer for a total of 30 minutes at 30°C. Oocytes are rinsed twice with 2XSSCT and washed 10 minutes in 2X SSCT. For simultaneous immunostaining, oocytes are washed in PBS and the immunostaining protocol can be resumed from blocking in PBS-T + 10% FCS followed by antibody incubation. With this method, it is possible to visualise the chromosomes (DAPI), the spindle (DM1A) and the centromere of the 3rd chromosome (dodeca satellite), showing bi-orientation of centromere 3 within the bipolar meiotic spindle (Zhaunova et al., 2016; Figure 2)

B. Live imaging of the spindle in oocytes

B.i) Imaging the bipolar spindle and FRAP analysis

Live-imaging in tissues such as oocytes is difficult in many aspects. Problems imposed by the thickness of the tissue can be bypassed using confocal microscopy, allowing the acquisition of a single focal plan with minimal out-of-focus signals. The sensitivity however is compromised compared to fixed oocytes as the oocyte is surrounded by a chorion that prevents light efficiently crossing the tissue. Photo-bleaching of the fluorescently-tagged proteins represents another problem and prevent long term laser exposure. Spinning Disk confocal microscopes allow faster acquisition with reduced photo-bleaching, but with higher background noise due to a leakage of out-of-focus signals. Caution is required to control for potential artefacts arising from the expression of a fluorescently-tagged protein.

As with immunostaining of oocytes, flies expressing fluorescently-tagged proteins of interest are matured for 3 to 5 days at 25°C to obtain late-stage oocytes (stage 13 and 14). They are then dissected in halocarbon oil 700 at room temperature, ovaries torn apart with forceps and oocytes spread out onto a coverslip (24 x 50mm) to separate early oogenesis stages from stage-13 or -14 oocytes. To image metaphase I spindles, stage 14 oocytes with long appendages are used (Gilliland et al., 2009; King, 1970). A commonly used chromosome marker, H2Av-RFP, is not suitable for long-term live-imaging due to rapid photobleaching. We developed a way to visualise the chromosomes and the spindle in living oocytes for long-term imaging by expressing RCC1-mCherry (Regulator of Chromatin Condensation 1) to mark the chromosomes (Colombié et al., 2013) and GFP- α -tubulin to mark microtubules (Beaven et al., 2017; Colombié et al., 2008; Głuszek et al., 2015) under the control of an oogenesis-specific Gal4 driver (*nanos-GAL4*, MVD1). This can be used to live-image metaphase I spindles with minimal spindle and chromosome defects when flies are matured at 18°C. 0.8 μ m Z-stacks of each channel are captured every 30-60 seconds.

Flies expressing GFP- α -tubulin and RCC1-mCherry can also be recombined with a large variety of RNAi lines/mutants to study the effect of gene depletion on spindle stability (Beaven et al., 2017; Colombié et al., 2013, Gluszek et al., 2015). It is possible to study the dynamics of the microtubules using FRAP (Fluorescent Recovery After Photo-bleaching) in different genetic backgrounds to analyse the effect of gene depletion on the spindle microtubule dynamics (Colombié et al., 2013). The localisation and dynamics of any other fluorescently-tagged proteins on the metaphase spindle can also be analysed with this method (Gluszek et al., 2015).

B.ii) From nuclear envelope breakdown to spindle bipolarity establishment and maintenance
It is also possible to study the process of meiotic spindle formation from the nuclear envelope breakdown (NEBD). Mature flies also contain some early stage-13 oocytes which have an intact nuclear envelope. They can be distinguished from stage-14 oocytes, as they have immature appendages in the anterior part of the oocyte (Gilliland et al., 2009; King, 1970). An intact nucleus can be recognised by accumulation of RCC1-Cherry signals in the nucleoplasm or exclusion of GFP- α -tubulin signal from the nucleus. Just before NEBD, GFP- α -tubulin is accumulated in the nucleus, and upon NEBD, GFP- α -tubulin and RCC1-Cherry diffuses in the ooplasm. This is followed by microtubule assembly around the chromosomes. 0.8 μ m Z-stack sections of each channel are captured every 30-60 seconds. Approximately 10 minutes after the NEBD the first microtubules start to appear around the chromosomes. Spindle bipolarity is roughly established ~30 minutes after the NEBD (Colombié et al., 2008; Gluszek et al., 2015: Figure 3). As *Drosophila melanogaster* lives at 18-25°C, it is possible to follow oocytes live at room temperature. From nuclear envelope breakdown to spindle bipolarity establishment/maintenance can be followed for a couple of hours or even longer, without significant photo-bleaching or loss of viability as evident by collapse of the spindle.

C. Biochemistry in ovaries

C.i) Western blot on ovaries and oocytes

Drosophila oocytes can be used for biochemical studies, due to the large size of their ovaries and the cell cycle arrest in mature oocytes. The simplest analysis is a western blot to detect a protein of interest. A single ovary provides enough material to visualise an endogenous protein of interest by western blot using an antibody specific to that protein. To avoid the unwanted action of endogenous phosphatases or proteases during protein preparation, 10 ovaries from matured flies are dissected in methanol, and transferred into a microfuge tube. The methanol is then replaced with 50 μ l of water. Alternatively, adult flies can be boiled with 50 μ l of water in a microfuge tube before dissection. It is also possible to manually separate oocytes/egg chambers and collect those at specific stages of oogenesis under a dissection microscope. When preserving phosphorylation or other sensitive modifications is crucial, mature females are dissected in methanol followed by separation of ovarioles at early or late stages (Cullen et al., 2005). Collected ovarioles are homogenised in 50 μ l of PBS with a plastic pestle in the microfuge tube. 50 μ l of 2X SDS sample buffer (100 mM Tris-HCl pH 6.8; 4% SDS; 20% glycerol; 0.2% Bromophenol Blue; 1.43M 2-mercaptoethanol) is added, before being boiled for 4 minutes to denature proteins. A sample equivalent to a single ovary is loaded on a gel for SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) followed by western blot.

C.ii) Immunoprecipitation to detect protein-protein interaction

Ovaries of matured flies are predominantly composed of stage-14 oocytes arrested in metaphase I. Therefore, it is possible to collect many synchronised oocytes to explore protein-protein interaction at this specific cell cycle stage. We can immunoprecipitate endogenous or ectopically expressed tagged proteins to identify their co-precipitated

partners. As little as 15 or as many as hundreds of matured females are dissected in PBS+2 mM EGTA. At this stage, ovaries can be transferred in a microfuge pre-chilled on dry ice, snap frozen in liquid nitrogen and stored at -80°C up to several months. Ovaries are resuspended in lysis buffer (20 mM Tris pH7.5, 50mM NaCl, 5mM EGTA, 1mM DTT, 1mM PMSF, 0.5% Triton + protease inhibitors). Ovaries are then crushed in lysis buffer using a glass tissue grinder (Dounce) and incubated for 30 minutes on ice. Ovary extracts are subsequently centrifuged at 16,000 g for 30 minutes at 4°C. Cellular debris (pellet) are discarded and soluble proteins (supernatant) are mixed with antibodies coupled to magnetic beads (for example, magnetic GFP-Trap®_M beads from chromotek; #gtm-20) for 1 hour to overnight at 4°C. For a pilot test, use 10 µl of slurry beads per 100 µl of lysate. The amount of beads can be optimised by checking the amount of targeted protein that remains in the unbound fraction after immunoprecipitation. Beads are then quickly washed three times with excess lysis buffer + 0.1% Triton and resuspended in boiling SDS sample buffer prior to further analysis. Note that the composition of the buffer used for washing the beads (salt concentration, detergent etc.) and the duration of the washes can be modified to obtain the desired stringency of the washes. Western blotting can be performed to investigate the presence of candidate interacting proteins or samples can be analysed by mass spectrometry to identify proteins co-precipitated with the protein of interest. It is essential to compare with controls, such as oocytes expressing the tag alone (GFP, FLAG, Myc etc), use of unrelated antibodies of identical or similar isotype, or use of magnetic beads alone.

III. References

Abad J.P., Carmena M., Baars S., Saunders R.D., Glover D.M., Ludeña P., Sentis C., Tyler-Smith C., Villasante A., 1992. Dodeca satellite: a conserved G+C-rich satellite from the centromeric heterochromatin of *Drosophila melanogaster*. *Proc Natl Acad Sci U S A*. 89 (10), 4663-7.

Bateman J.R., Lee A.M., Wu C.T., 2006. Site-specific transformation of *Drosophila* via phiC31 integrase-mediated cassette exchange. *Genetics* 173 (2), 769-77.

Beaven R., Bastos R.N., Spanos C., Romé P., Cullen C.F., Rappsilber J., Giet R., Goshima G., Ohkura H., 2017. 14-3-3 regulation of Ncd reveals a new mechanism for targeting proteins to the spindle in oocytes. *J Cell Biol.* 216 (10), 3029-3039.

Bischof J., Maeda R.K., Hediger M., Karch F., Basler K., 2007. An optimized transgenesis system for *Drosophila* using germ-line-specific phiC31 integrases. *Proc Natl Acad Sci U S A*. 104 (9), 3312-7.

Bloch Qazi M.C., Heifetz Y., Wolfner M.F., 2003. The developments between gametogenesis and fertilization: ovulation and female sperm storage in *Drosophila melanogaster*. *Dev Biol.* 256 (2), 195-211.

Carmena M., Abad J.P., Villasante A., Gonzalez C., 1993. The *Drosophila melanogaster* dodecasatellite sequence is closely linked to the centromere and can form connections between sister chromatids during mitosis. *J Cell Sci.* 105 (Pt 1), 41-50.

Colombié N., Cullen C.F., Brittle A.L., Jang J.K., Earnshaw W.C., Carmena M., McKim K., Ohkura H., 2008. Dual roles of Incenp crucial to the assembly of the acentrosomal metaphase spindle in female meiosis. *Development* 135(19), 3239-46.

Colombié N., Głuszek A.A., Meireles A.M., Ohkura H., 2013. Meiosis-specific stable binding of augmin to acentrosomal spindle poles promotes biased microtubule assembly in oocytes. *PLoS Genet.* 9 (6), e1003562.

Cullen C.F., Ohkura H., 2001. Msps protein is localized to acentrosomal poles to ensure bipolarity of *Drosophila* meiotic spindles. *Nat Cell Biol.* 3 (7), 637-42.

Cullen C.F., Brittle A.L., Ito T., Ohkura H., 2005. The conserved kinase NHK-1 is essential for mitotic progression and unifying acentrosomal meiotic spindles in *Drosophila melanogaster*. *J Cell Biol.* 171(4), 593-602.

Dernburg A.F., Sedat J.W., Hawley R.S., 1996. Direct evidence of a role for heterochromatin in meiotic chromosome segregation. *Cell.* 86 (1), 135-46.

Dernburg A.F., 2000. In situ hybridization to somatic chromosomes. In Sullivan W., Ashburner M., Hawley R.S. editor: *Drosophila protocols*, New York, 2000, Cold Spring Harbor Laboratory Press, p25-55.

Fish M.P., Groth A.C., Calos M.P., Nusse R., 2007. Creating transgenic *Drosophila* by microinjecting the site-specific phiC31 integrase mRNA and a transgene-containing donor plasmid. *Nat Protoc.* 2 (10), 2325-31.

Fischer J.A., Giniger E., Maniatis T., Ptashne M., 1988. GAL4 activates transcription in *Drosophila*. *Nature* 332 (6167), 853-6.

Gilliland W.D., Hughes S.F., Vietti D.R., Hawley R.S., 2009. Congression of achiasmate chromosomes to the metaphase plate in *Drosophila melanogaster* oocytes. *Dev Biol.* 325 (1), 122-8.

Gluszek A.A., Cullen C.F., Li W., Battaglia R.A., Radford S.J., Costa M.F., McKim K.S., Goshima G., Ohkura H., 2015. The microtubule catastrophe promoter Sentin delays stable kinetochore-microtubule attachment in oocytes. *J Cell Biol.* 211 (6), 1113-20.

Groth A.C., Calos M.P., 2004. Phage integrases: biology and applications. *J Mol Biol.* 335 (3), 667-78.

Groth A.C., Fish M., Nusse R., Calos M.P., 2004. Construction of transgenic *Drosophila* by using the site-specific integrase from phage phiC31. *Genetics* 166 (4), 1775-82.

Jang J.K., Rahman T., McKim K.S., 2005. The kinesin like protein Subito contributes to central spindle assembly and organization of the meiotic spindle in *Drosophila* oocytes. *Mol Biol Cell* 16 (10), 4684-94.

Karsenti E. and Vernos I., 2001. The Mitotic Spindle: A Self-Made Machine. *Science* 294 (5542), 543-547.

King R.C., 1970. The meiotic behavior of the *Drosophila* oocyte. *Int Rev Cytol.* 28, 125-68.

Kirschner M. and Mitchison T., 1986. Beyond self-assembly: From microtubules to morphogenesis. *Cell* 45(3), 329-42.

Loh B.J., Cullen C.F., Vogt N., Ohkura H., 2012. The conserved kinase SRPK regulates karyosome formation and spindle microtubule assembly in *Drosophila* oocytes. *J Cell Sci.* 125 (Pt 19), 4457-62.

McKim K.S., Hawley R.S., 1995. Chromosomal control of meiotic cell division. *Science* 270 (5242), 1595-601.

Meireles A.M., Fisher K.H., Colombié N., Wakefield J.G., Ohkura H., 2009. Wac: a new Augmin subunit required for chromosome alignment but not for acentrosomal microtubule assembly in female meiosis. *J Cell Biol.* 184 (6), 777-84.

Ni J.Q., Zhou R., Czech B., Liu L.P., Holderbaum L., Yang-Zhou D., Shim H.S., Tao R., Handler D., Karpowicz P., Binari R., Booker M., Brennecke J., Perkins L.A., Hannon G.J., Perrimon N., 2011. A genome-scale shRNA resource for transgenic RNAi in *Drosophila*. *Nat Methods* 8 (5), 405-7.

Ohkura H., 2015. Meiosis: an overview of key differences from mitosis. *Cold Spring Harb Perspect Biol.* 7 (5).

Page AW, Orr-Weaver TL, 1997. Stopping and starting the meiotic cell cycle. *Curr Opin Genet Dev.* 7 (1), 23-31.

Radford S.J., Jang J.K., McKim K.S., 2012. The chromosomal passenger complex is required for meiotic acentrosomal spindle assembly and chromosome biorientation. *Genetics* 192 (2), 417-29. *Chromosoma* 126 (3), 351-364.

Radford S.J., Nguyen A.L., Schindler K. McKim K.S., 2017. The chromosomal basis of meiotic acentrosomal spindle assembly and function in oocytes. *Chromosoma*. 126 (3), 351-364.

Rørth, 1998. Gal4 in the *Drosophila* female germline. *Mech Dev*. 78 (1-2), 113-8.

Staller M.V., Yan D., Randklev S., Bragdon M.D., Wunderlich Z.B., Tao R., Perkins L.A., Depace A.H., Perrimon N., 2013. Depleting gene activities in early *Drosophila* embryos with the "maternal-Gal4-shRNA" system. *Genetics* 193(1), 51-61.

Tavosanlis G., Llamazares S., Goulielmos G., Gonzalez C., 1997. Essential role for gamma-tubulin in the acentriolar female meiotic spindle of *Drosophila*. *EMBO J*. 16 (8), 1809-19.

Theurkauf W.E. and Hawley R.S., 1992. Meiotic spindle assembly in *Drosophila* females: behavior of nonexchange chromosomes and the effects of mutations in the nod kinesin-like protein. *J Cell Biol*. 116 (5), 1167-80.

Zhaunova L., Ohkura H., Breuer M., 2016. Kdm5/Lid Regulates Chromosome Architecture in Meiotic Prophase I Independently of Its Histone Demethylase Activity. *PLoS Genet*. 12 (8), e1006241.

Figure 1. Immunostaining of meiotic spindles in control and 14-3-3 ϵ -depleted oocytes.

Oocytes expressing shRNA against *white* gene (control) or *14-3-3 ϵ* gene are fixed and immunostained. DNA (blue) is stained with DAPI and α -tubulin (green) is stained with DM1A antibody (mouse monoclonal DM1A, Sigma-Aldrich; Alexa Fluor 488-conjugated anti-mouse secondary antibody). TACC (rabbit polyclonal D-TACC-CTD, Loh et al., 2012; Cy3-conjugated anti-rabbit secondary antibody; magenta, left panel) decorates the poles of the meiotic spindle while Subito (rat polyclonal Subito, Loh et al., 2012; Cy3-conjugated anti-rat secondary antibody; magenta, right panel), decorates the equatorial region of the spindle. In 14-3-3 ϵ depleted oocytes, the meiotic spindle is abnormal, often displaying a tripolar shape as opposed to the normal bipolar spindle observed in control oocytes. However, TACC and Subito localisation are not affected by the loss of 14-3-3 ϵ . Scale bar, 10 μ m. (adapted from Beaven et al., 2017)

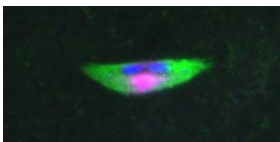
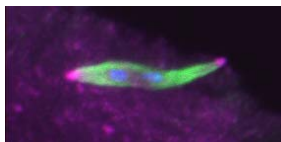
Figure 2. FISH and immunostaining of meiotic spindles in control and *Lid*-depleted oocytes.

Oocytes expressing shRNA against *white* gene (control) or *lid* gene are processed for FISH probed by the dodeca satellite sequences (Cen3), followed by immunostaining. DNA (magenta) is stained with DAPI, and α -tubulin is stained with DM1A antibody (mouse monoclonal DM1A, Sigma-Aldrich; Alexa Fluor 488-conjugated anti-mouse secondary antibody). This probe (green; Alexa Fluor 546-dUTP) recognises the dodeca satellite sequences specific to the peri-centromeric region of the third chromosome that appear as two foci, one for each homologue of centromere 3. In *Lid*-depleted oocytes, chromosomes are split into two distinct clusters compared to the normal single chromosome cluster observed in control oocytes. Contrary to the bioriented Cen3 foci pointing out towards opposite spindle poles observed in control oocytes (yellow arrows, top), *lid* RNAi oocytes often display monopolar orientation of the chromosomes. This is highlighted by the proximity of the two Cen3 foci to the same side of the spindle (yellow arrow, bottom). Scale bar, 5 μ m. (adapted from Zhaunova et al., 2016)

Figure 3. Live-imaging of meiotic spindle formation from microtubule assembly to bipolarity establishment. Oocytes from females expressing GFP- α -tubulin (microtubules, green) and Rcc1-mCherry (chromosomes, red) in a wild-type background (wild type) or in an Augmin mutant background (*wac* Δ) are imaged from the nuclear envelope breakdown (NEB= 00:00) to the establishment of a bipolar meiotic spindle (25:00). Augmin depletion does not perturb spindle assembly, nor the timing of bipolarity establishment. However, Rcc1-mCherry reveals that chromosomes fail to congress into a spherical structure and appear spread out along the spindle when Augmin is absent. Time is indicated in min:sec; scale bar, 10 μ m. (adapted from Colombié et al., 2013).

TACC tubulin DNA Subito tubulin DNA

control



14-3-3ε RNAi

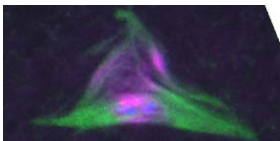
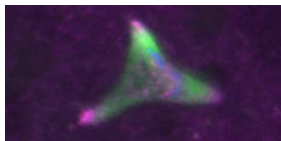


Figure 1

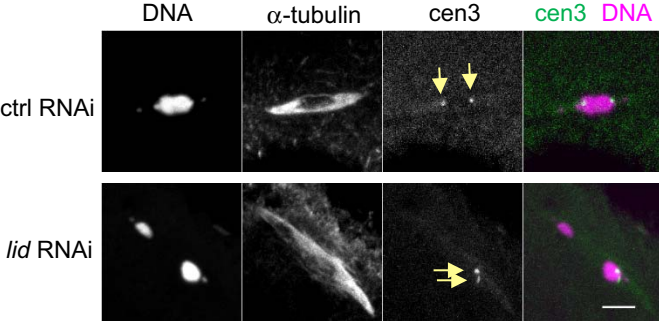


Figure 2

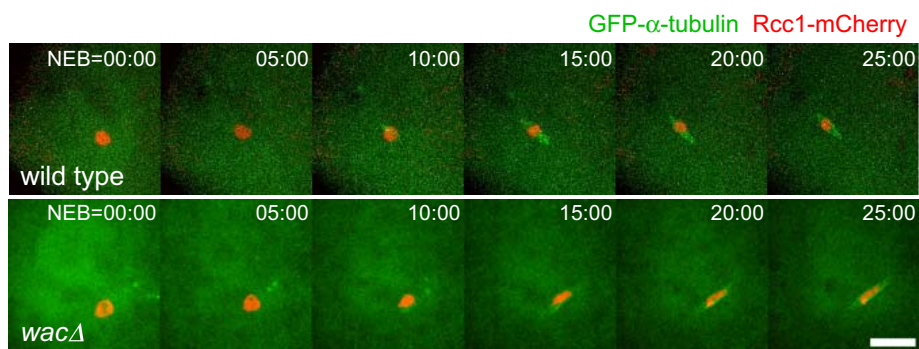


Figure 3